

In re Appln. of Feussner et al.
Application No. Unassigned (U.S. National Phase of PCT/EP00/06539)

A3 Figure 3 shows the sequence of wild type LOX of potato tubers [SEQ ID NO:3]. The mutagenized amino acid position is underlined. Primers 1 and 2 [SEQ ID NOS: 1 and 2, respectively] as used are also shown.

[Replace the paragraph beginning at page 3, line 8, with:]

In a preferred embodiment, the amino acids are changed in the region of the amino acid position 570 to 581 of potato tuber LOX. The above-indicated amino acid positions refer to the sequence under the access number S73865 in the EMBL data base or the sequence according to Fig. 3. The positions in LOXs of other plant species, which correspond to the amino acid positions 593 to 602 of *Cucumis sativus* lipoxygenase, can easily be determined by sequence comparisons between sequence X92890 and the further protein sequences, e.g., of soybean, potato, arabidopsis, tobacco or barley. The following Table 1 shows the result of an amino acid comparison between the cucumber-derived enzyme and the corresponding positions in the enzymes of other plants. The first group (15-LOX) shows a comparison between LOXs which at position 15 introduce a hydroperoxy group into an arachidonic acid molecule, while the second group (5-LOX) shows a comparison between sequences which introduce a hydroperoxy group at position 5.

Replace the paragraph beginning at page 5, line 1, with:

A4 The present invention further relates to LOX mutants which are obtainable according to the above-described methods. The LOXs according to the invention can be produced with the help of the methods known from the prior art, for example directed mutagenesis, and subsequent protein expression. In particular mutants which after incubation with arachidonic acid yield at least 40%, preferably 50%, of the derivative perhydroxylated at position 11 are considered to be inventive.

Replace the paragraph beginning at page 5, line 27, with:

A5 Finally, new plants or plant parts can be regenerated from the above-mentioned cells by *in vitro* culturing methods. For the production of such transgenic plants the known transformation system can be used, e.g., on the basis of *Agrobacteria* and Ti plasmid derivatives.

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Replace the paragraph beginning at page 6, line 4, with:

A6
Particularly preferred is an arachidonic acid derivative which contains a hydroperoxy group at position 11. The derivative can then easily be converted into the hydroxy derivative. The thus available 11S-HPETE can be used for producing the alcohols, aldehydes and dicarbonic acids shown below. The enzyme hydroperoxide lyase is contained in extracts of cucumber seedlings, for example. 2E- and 3Z-nonenal and their alcohols are important flavorings in foodstuff (e.g., cucumbers).

Replace the paragraph beginning at page 7, line 15, with:

A7
For bacterial expression of wild type LOX and LOX mutant and for directed mutagenesis, use was made of the plasmid pet3b (Novagen, Germany) which contained the cDNA of the potato tuber LOX as insert (pET-LOX1; cf. Geerts, A., Feltkamp, D., Rosahl, S. (1994) Expression of lipoxygenase in wounded tubers of *solanum tuberosum* L., Plant Physiol. 105: 269-277). Mutagenesis was carried out by using the QuikChange Mutagenesis Kit from Stratagene (Heidelberg, Germany). Oligonucleotides containing the appropriate base exchanges were purchased from MWG-Biotech (Ebersberg, Germany). To analyze the mutation, an additional conservative base exchange was introduced to construct a new restriction cleavage site. In addition, the mutation was sequenced and at least five different bacterial clones were expressed and used for analyzing the enzymatic characteristics. Expression of pET-LOX1 and its mutant was performed as described by Feussner, I., Bachmann, A., Höhne, M. & Kindl, H. (1998) FEBS Lett. 431, 433-436. Cells from 1 liter cultures were resuspended in 5-7 ml lysis buffer and disrupted by using a sonifier tip with pulses each of 30 seconds, and cellular debris was pelleted.

Replace the paragraph beginning at page 8, line 4, with:

A8
For product analysis, 0.9 ml of cell lysates were incubated with 0.9 mM arachidonic acid (final concentration) in 100 mM Tris buffer, pH 7.5, for 30 minutes at room temperature. Reaction was stopped by the addition of sodium borohydride to convert the hydroperoxy fatty acids formed to the corresponding hydroxy compounds. The samples were acidified to pH 3 and the lipids were extracted (cf. Bligh, E.G. & Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911-917). The lower chloroform phase was recovered and the solvent was evaporated. The remaining lipid was dissolved with 0.1 ml methanol, and aliquots were subjected to HPLC analysis.

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Replace the paragraph beginning at page 8, line 15, with:

AG HPLC analysis was carried out on a Hewlett Packard 1100 HPLC system coupled to a diode detector. RP-HPLC of the free fatty acid derivatives was carried out on a Nucleosil C-18 column (Macherey-Nagel, 250 x 4 mm, 5µm particle size) with a solvent system of methanol/water/acetic acid (85/15/0.1; v/v/v) and at a flow rate of 1 ml/min. Absorption at 234 nm (absorption of the conjugated diene system of the hydroxy fatty acids) and at 210 nm (polyenoic fatty acids) was recorded accordingly. Straight-phase HPLC (SP-HPLC) of hydroxy fatty acid isomers was carried out on a Zorbax SIL column (HP, Waldbronn, Germany; 250 x 4.6 mm, 5 µm particle size) with a solvent system of n-hexane/2-propanol/acetic acid (100/2/0.1, v/v/v) at a flow rate of 1ml/min. The enantiomer composition of the hydroxy fatty acids was analyzed by chiral-phase HPLC on a Chiralcel OD column (Daicel Chem. Industries, distributed by Baker Chem., Deventer, Netherlands; 250 x 4.6 mm, 5µm particle size) with a solvent system of hexane/2-propanol/acetic acid (100/5/0.1, v/v/v) at a flow rate of 1 ml/min. (Cf. Feussner, I., Balkenhohl, T.J., Porzel, A., Kühn, H.& Wasternack, C. (1997) J. Biol. Chem. 272, 21635-21641).

Replace the paragraph beginning at page 9, line 10, with:

AG¹⁰ The starting cDNA and the mutagenesis kit were as described above. For analysis of the mutation further conservative base exchanges were carried out for producing a new restriction cleavage site for BsTBL. The following primers were used for producing the mutation V576F: GCT GGT GGG GTT CTT GAG AGT ACA TTC TTT CCT TCG AAA TTT GCC ATG GAA ATG TCA GCT G (coding strand) [SEQ ID NO:1] and CAG CGT ACA TTT CCA TGG CAA ATT TCG AAG GAA AGA ATG TAC TCT CAA GAA CCC CAC CAG C (complementary strand) [SEQ ID NO:2]. Furthermore, the mutant was sequenced and 5 different bacterial colonies were expressed and used for enzymatic studies. The expression of pET-LOX1 was carried out as described above. The further preparation was carried out as already indicated above. Analysis of the produced fatty acid derivative (containing a hydroperoxy group at position 11) was carried out as indicated above. The result of the SP-HPLC analysis for converting arachidonic acid with V576F is shown in Fig. 2. The following Table 2 shows a comparison of the specificity of the wild type (wtLOX) with the mutant (LOXV_{576F}).